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Human CAP18: a Novel Antimicrobial Lipopolysaccharide-Binding Protein

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CAP18 (18-kDa cationic antimicrobial protein) is a protein originally identified and purified from rabbit leukocytes on the basis of its capacity to bind and inhibit various activities of lipopolysaccharide (LPS). Here we report the cloning of human CAP18 and characterize the anti-LPS activity of the C-terminal fragment. Oligonucleotide probes designed from the rabbit CAP18 cDNA were used to identify human CAP18 from a bone marrow cDNA library. The cDNA encodes a protein composed of a 30-amino-acid signal peptide, a 103-amino-acid N-terminal domain of unknown function, and a C-terminal domain of 37 amino acids homologous to the LPS-binding antimicrobial domain of rabbit CAP18, designated $CAP18_{104-140}$. A human CAP18-specific antiserum was generated by using CAP18 expressed as a fusion protein with the maltose-binding protein. Western blots (immunoblots) with this antiserum showed specific expression of human CAP18 in granulocytes. Synthetic human $CAP18_{104-140}$ and a more active truncated fragment, $CAP18_{104-135}$, were shown to (i) bind to erythrocytes coated with diverse strains of LPS, (ii) inhibit LPS-induced release of nitric oxide from macrophages, (iii) inhibit LPS-induced generation of tissue factor, and (iv) protect mice from LPS lethality. $CAP18_{104-140}$ may have therapeutic utility for conditions associated with elevated concentrations of LPS.

Lipopolysaccharide (LPS), a major constituent of the gram-negative bacterial outer membrane, is implicated in the pathogenesis of sepsis, a syndrome that accounts for >2% of all hospital admissions and >400,000 deaths annually (5, 29). Although antibiotics kill bacteria, they do not neutralize LPS, and they may potentiate bacterial release of LPS with acceleration of the inflammatory process (41). LPS activates macrophages and endothelial cells, stimulating the release of potent inflammatory mediators such as tumor necrosis factor (TNF) and free radicals (4, 28). Cationic antibiotics such as polymyxin B bind to and neutralize some types of LPS; however, clinical use is limited by toxicity (44, 50), despite efforts to generate nontoxic congeners (50). Other endotoxin-neutralizing strategies include the use of antibodies (7, 8, 58) and various endotoxin-binding factors from *Limulus* spp. and mammals (6, 48).

The most intensely studied mammalian-derived endotoxin-binding factors are an acute-phase protein produced by the liver called LPS-binding protein (LBP) (37, 45, 46) and a polymorphonuclear leukocyte granule protein called bactericidal permeability-increasing protein (BPI; CAP57) (10, 11, 40). LPS complexed to LBP binds to the macrophage surface protein CD14 (54, 55) and is 100-fold more potent than LPS alone in triggering various monocyte responses such as synthesis of TNF. In contrast, BPI appears to function as part of the neutrophil's arsenal of antibacterial peptides. When granulocytes are attracted to sites of infection, they engulf gram-negative bacteria. BPI is located membrane bound inside the azurophilic granules and is therefore well situated to bind LPS released from phagocytosed bacteria. Furthermore, recent studies demonstrate that binding of BPI to lipid A inhibits many of the cellular responses to LPS (14, 26, 51). Accordingly, recombinant BPI and the 23-kDa N-terminal LPS-binding fragment of BPI are under clinical evaluation for treatment of sepsis (51, 53).

Rabbit granulocytes contain an 18-kDa cationic protein with microbicidal and LPS-binding properties. We have purified and characterized this protein, called CAP18, using as an assay its capacity to agglutinate sheep erythrocytes sensitized with LPS (16–18, 20–22, 48). This is a nonspecific assay, and we have corroborated the LPS-binding and antimicrobial activities of the molecule seen in several other systems (17, 20, 21). In the present paper we describe the cloning of human CAP18 and demonstrate that it is expressed in normal human granulocytes and that the C-terminal portion of human CAP18 binds to LPS, neutralizes LPS-mediated activation of monocytes, and protects mice injected with lethal quantities of LPS.

MATERIALS AND METHODS

LPS. LPSs were purchased from List Biologicals (Campbell, Calif.) unless indicated otherwise.

Erythrocyte agglutination assay. One milliliter of 1% erythrocytes (human O type, C3H/HeN mouse, or sheep) was sensitized by incubation with 0.2 ml of Re-LPS (*Salmonella minnesota* R595) solution (100 μg/ml in phosphate-buffered saline [PBS]) at 37°C for 30 min, followed by washing with PBS (17). Fifty microliters of a 1.0% suspension of sensitized erythrocytes was mixed with 50 μl of a twofold serial dilution of CAP18 or CAP18 peptides in a U-bottom microtiter plate and incubated at 37°C for 1 h. Activity of CAP18 was expressed as the minimum agglutinating concentration.

Cloning of human CAP18 cDNA. (i) Rabbit CAP18 cDNA. Details of the cDNA cloning of rabbit CAP18 have been published previously (22). The sequence is listed in GenBank under accession no. M73998. The cDNA encodes a mature protein of 142 amino acids with a conventional 29-amino-acid signal sequence (see Fig. 1). The sequence and hydropathic profile of this novel protein distinguish it from other LPS-binding proteins such as LBP and BPI. The predicted molecular mass is 16.6 kDa. The predicted pI is 10. There are no asparagine-linked carbohydrate attachment sites.

(ii) Human CAP18 cDNA. Numerous attempts to identify human CAP18 by using PCR primers designed from the C-terminal 37 amino acids of rabbit CAP18 were unsuccessful. Subsequently, duplicate filters were prepared from a million plaques and controls of a lambda gt11 human bone marrow cDNA library (purchased from Clontech Laboratories, Palo Alto, Calif.). One set of filters was

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screened with the rabbit CAP18 cDNA, and the other was screened with a $CAP_{104-149}$ probe. Both probes were labelled with ^{32}P by PCR. No positives were detected among a total of 500,000 plaques screened at standard stringency with either probe. However, by using the CAP18 probe 14 putative positives were detected among a total of 500,000 plaques screened under reduced stringency. In this case the temperature and salt concentration were kept constant and the formamide concentration was reduced from 50 to 30% during prehybridization and hybridization, with washing at 50°C instead of 65°C. Six of the most intense positives were chosen for a second round of screening, and four of these yielded unequivocal individual positive plaques. Lambda DNA was purified, and the inserts were cloned into TA vectors for dideoxy sequencing. One of these was a false positive, whereas three were bona fide CAP18 cDNAs of slightly different lengths. The 5′ end of the mRNA was determined by using the 5′-RACE system from Life Technologies Inc. (Gaithersburg, Md.).

CAP18 protein studies. (i) Generation of antibodies. The human CAP18 cDNA was cloned as a fusion protein with the *Escherichia coli* maltose-binding protein at the N terminus (New England BioLabs, Beverly, Mass.). The fusion protein was affinity purified by using an amylose column and maltose elution. Rabbits were immunized with fusion protein (100 μg) in complete Freund's adjuvant and given three boosters of protein (100 μg) in incomplete Freund's adjuvant. Serum was collected, and antibodies were purified by using protein A-Sepharose with pH 2.8 elution. Purified antibodies were dialyzed against PBS, concentrated, and stored at −20°C. As determined by solid-phase enzyme-linked immunosorbent assay using wells coated with fusion protein, the purified immune sera had a titer of $1:10^7$.

(ii) Preparation of cells. U937 (human histiocytic lymphoma), L1210 (mouse leukemia), HL60 (human promyelocyte), SMMC (human hepatoma), and Cos 7 (African green monkey kidney) cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mg of glutamine per ml. All cell lines were documented to be mycoplasma free prior to use. Buffy coats were obtained from the Stanford University blood bank, and various leukocyte subpopulations were separated by using Histopaque-1119 and -1077 (Sigma). Granulocytes were collected directly from the gradients. Other leukocytes were cultured overnight to separate nonadherent lymphocyte and adherent monocyte subpopulations. Populations were >95% pure as determined by microscopic observation.

(iii) Western blots (immunoblots). Approximately 5×10^7 cells (in 50 μl of PBS) from each population were resuspended in 50 μl of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Cells were boiled for 10 min, and DNA was fragmented by suspension with a 26-gauge needle. Cells were spun (11,000 × *g*) for 10 min, and 20 μl of soluble protein supernatant was applied to an SDS–12% PAGE gel. After electrophoresis, separated proteins were blotted onto nitrocellulose membranes and then blocked with 5% "BLOTTO-nonfat dried milk" in 50 mM Tris-HCl (pH 7.5)–200 mM NaCl–0.05% Tween 20 buffer (TBST). Blots were exposed to immune (anti-CAP18) or preimmune serum diluted 1:800 in TBST for 2 h and then washed twice for 15 min in TBST at room temperature. Next, blots were exposed to an affinity-purified biotinylated goat anti-rabbit antiserum diluted 1:1,000 in TBST for 1 h and washed twice for 15 min in TBST. Next, blots were exposed to streptavidin-conjugated alkaline phosphatase diluted 1:12,000 in TBST and were washed twice for 15 min in Tris-buffered saline and once in alkaline phosphatase substrate buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-Cl [pH 9.5]). Finally the blots were exposed to 25 ml of alkaline phosphatase substrate buffer containing 110 μl of nitroblue tetrazolium (50 mg/ml in 70% dimethylformamide) and 83 μl of 5-bromo-4-chloro-3-indolylphosphate (50 mg/ml in 70% dimethylformamide). After 10 to 20 min, 400 μl of 0.5 M EDTA was added to stop the enzyme reaction and the blots were air dried prior to photography.

Assay of RNI. The murine macrophage cell line RAW 264.7 (obtained from the American Type Culture Collection) and thioglycolate-elicited murine peritoneal exudate cells were used to produce reactive nitrogen intermediates (RNI). Cells were cultured at 10^6/ml in RPMI 1640 plus 2.5% fetal calf serum in 24-well plates in the presence or absence of different concentrations of *E. coli* O111:B4 LPS mixed with various concentrations of CAP18 peptides. In some experiments, mouse gamma interferon (purchased from Sigma Chemical Company) was used to activate production of RNI. After 24 h of incubation at 37°C, the cell-free supernatant was collected and tested for the presence of RNI. Accumulation of nitrite in the medium was measured by a colorimetric assay based on the Griess reaction (15) with sodium nitrite standards. Sample (50 μl) was mixed with 50 μl of Griess reagent (1% sulfanilamide, 0.1% naphthalene diamine dihydrochloride, 2.5% H_3PO_4), and after 10 min at room temperature the A_{570} was read.

Tissue factor assay. *S. minnesota* smooth LPS was incubated with various concentrations of each peptide for 5 min, and then the mixture was added to peritoneal murine macrophages obtained 4 days after thioglycolate stimulation (details of this assay were previously published [17]). Cells were cultured with LPS with or without CAP18 peptides for 6 h at 37°C in a 5% CO_2 incubator. The cell suspension was centrifuged, and the cell pellet was frozen at −80°C until clotting assays were performed. Tissue factor activity in modified unactivated partial thromboplastin time was tested. Cell lysate (10^6 cells per 100 μl) was preincubated with 100 μl of mouse plasma at 37°C for 3 min. Then 100 μl of 25 mM $CaCl_2$ containing phospholipid was added to the mixture and the clotting time was measured with a Fibrometer (BioQuest Division, Becton Dickinson, Cockeysville, N.C.).

Preparation of peptides. Peptides were synthesized, purified, and characterized as previously described (21).

Mouse LPS-induced lethality assays. (i) Galactosamine-sensitized mouse model. A galactosamine-sensitized mouse model assay was performed as described by Galanos et al. (13). C57BL/6 mice (males; 8 to 12 weeks of age; Charles River) were injected intraperitoneally (i.p.) with 15 mg of galactosamine alone or 15 mg of galactosamine plus 0.1 μg of smooth LPS from *S. minnesota*. Equal volumes of 100 μl of LPS and CAP18 peptides were mixed and incubated for 30 min at 37°C prior to injection. Survival over 1 week was recorded.

(ii) Actinomycin D-sensitized mouse model. An actinomycin D-sensitized mouse model assay was performed as described by Piereni et al. (31). Briefly, 10-fold dilutions of *E. coli* O111:B4 LPS in pyrogen-free saline or saline alone were incubated 1:1 with 20 μg of $CAP18_{104-149}$ per ml for 30 min at 37°C. One hundred microliters of each of these solutions together with 100 μl of a solution of 250 μg of actinomycin D per ml was injected i.p. into groups of 7 to 11 ddY mice (male; 10 to 12 weeks of age). Thus, all mice received 1.0 μg of CAP18 peptides, 25 μg of actinomycin D, and dilutions of LPS or saline. Results were recorded as numbers of survivors per total numbers of mice at 1 week.

Statistical analysis. Results are expressed as means ± standard deviations for at least three or four samples. Student's *t* test for unpaired data was used to determine statistical significance. A two-tailed *P* value of <0.05 was considered significant. Fifty percent inhibitory concentrations were determined by least-squares linear regression. The 50% lethal dose was calculated by the method of Reed and Muench (34).

Nucleotide sequence accession number. The human CAP18 cDNA sequence has been deposited in GenBank under accession no. U19970.

RESULTS

Identification of human CAP18. The human CAP18 cDNA was identified and sequenced as described in Materials and Methods (see Fig. 1 and 2). Translation of the cDNA revealed a protein with a conventional 30-amino-acid signal sequence. Like rabbit CAP18 the mature protein has two domains: an amino-terminal domain with a high level of homology to other known members of the CAP18 gene family and a carboxy-terminal endotoxin-binding domain with less homology. Figure 1 shows a comparison of the cDNAs of human and rabbit CAP18s. Figure 2 shows a comparison of the protein sequences of rabbit CAP18, human CAP18, and cathelin, a pig homolog of CAP18 (35). The 5′ untranslated region immediately adjacent to the ATG has the sequence 5′-GCAGACAT GGGGACC. Like that of another member of the CAP18 family (56), this 5′ untranslated region is unusually short. There is a much higher level of nucleic acid and amino acid conservation in the N-terminal domain compared with the carboxy-terminal LPS-binding domain. The amino acid identities of human, cow, pig, and rabbit CAP18s are shown in Table 1. The overall nucleic acid identities between both domains of the human and rabbit molecules are similar, 73 to 74%. However, for translated amino acids the N-terminal domains share 63% identity, whereas the C-terminal domains share only 38% identity.

The LPS-binding and antimicrobial fragment of rabbit CAP18 corresponded to a C-terminal 37-amino-acid fragment. The corresponding homologous human CAP18 peptide was synthesized for in-depth study.

Human CAP18 protein is specifically expressed in granulocytes. A high-titer rabbit anti-human CAP18 antiserum was generated by using a CAP18-*E. coli* maltose-binding fusion protein as an immunogen. This antiserum was used to localize the cellular source of CAP18 by Western blotting (Fig. 3). The antiserum correctly identified the fusion protein (molecular mass, 48 kDa) used as the immunogen (positive control [lane 9]). Bands corresponding to holo-CAP18 protein (molecular mass, 18 kDa) and the slightly smaller N-terminal domain (molecular mass, 14 kDa) of CAP18 (17) were detected in purified granulocytes but not in monocytes or lymphocytes, a result previously observed with unseparated rabbit peritoneal exudate cells (17). CAP18 protein was not detected in various

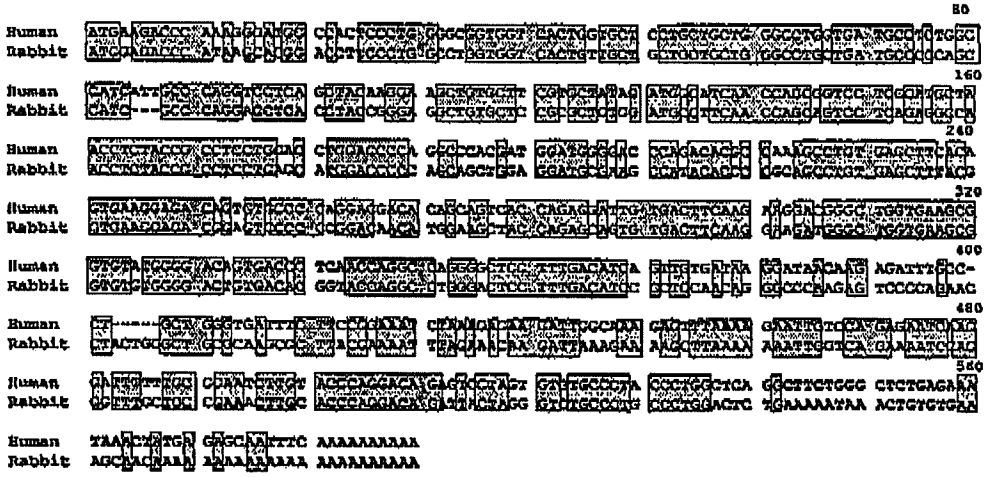

FIG. 1. Comparison of human and rabbit CAP18 cDNA sequences. The overall DNA sequence identity is ~73%. The sequences begin with the initiation ATG. The human coding sequence begins at position 90 (CAG; Gln). The stop codon for human CAP18 is at position 510.

cell lines, including LJ210, SMMC hepatoma, Cos 7, and most notably monocytic U937 and promonocytic HL60.

Synthetic human CAP18$_{104-140}$ peptides inhibit bioactivities of LPS in vitro. (i) LPS binding activity: agglutination of LPS-sensitized erythrocytes by synthetic human CAP18 peptides. Previous studies showed that rabbit CAP18 was composed of two domains: a highly conserved N-terminal domain with an unknown function and a much less conserved C-terminal domain with anti-LPS and antimicrobial functions (17, 21). These studies also identified a more active fragment corresponding to rabbit CAP18$_{106-137}$. Therefore, we studied the full-length C-terminal fragment of human CAP18, CAP18$_{104-140}$, and a truncated version, human CAP18$_{104-135}$. The capacities of these human peptides to agglutinate LPS-sensitized erythrocytes are compared in Table 2. As was the case for the rabbit peptides, the truncated human peptide (CAP18$_{104-135}$) was more active than the full-length human peptide CAP18$_{104-140}$. The functional activities of these two peptides were compared in several of the assays described below.

(ii) Synthetic human CAP18$_{104-140}$ inhibits LPS-induced RNI production. Low concentrations of LPS stimulate mouse macrophage RAW 264.7 cells to produce substantial quantities of RNI (21). Figure 4 shows a representative experiment (of three) demonstrating the capacity of synthetic rabbit CAP18$_{106-137}$ and human CAP18$_{104-135}$ to inhibit release of RNI from RAW 264.7 cells stimulated with 2.5 ng of LPS per ml. The 50% inhibitory concentration was less than 50 nM. Control experiments demonstrated that one of the inactive

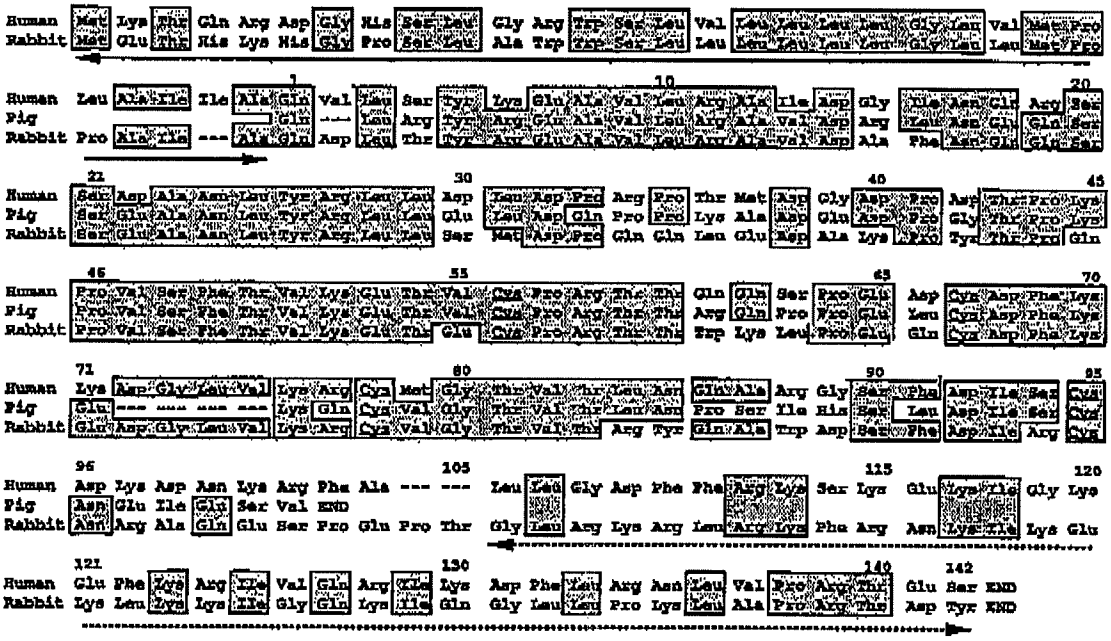

FIG. 2. Comparison of amino acid sequences of human, pig, and rabbit CAP18 family members. ◄——►, signal peptide; ◄——►, LPS-binding domain.

TABLE 1. Identical amino acids in the N-terminal domains of CAP18 family members

Protein	% of N-terminal amino acids identical to N-terminal amino acids of:				
	Human CAP18	Pig CAP18	Cow CAP18	Rabbit CAP18	p15H
Human CAP18	100				
Pig CAP18	61.6	100			
Cow CAP18	58.4	78.9	100		
Rabbit CAP18	63.1	64.2	59.4	100	
p15H	31.3	34.4	33.3	31.3	100

TABLE 2. LPS-binding activities of synthetic human and rabbit CAP18-derived amino-terminal peptides

Source	Sequence	LPS-binding activity (MAC [μg/ml])[a]
Rabbit	rabCAP18$_{106-142}$	4.2
	rabCAP18$_{106-137}$	2.1
Human	huCAP18$_{104-140}$	12.1
	huCAP18$_{104-135}$	1.6

[a] MAC, minimal agglutinating concentration for *S. minnesota* Re-LPS-sensitized sheep erythrocytes. Data are averages of those from two experiments.

cationic fragments of rabbit CAP18$_{106-142}$ (CAP18$_{122-142}$) did not block LPS-induced nitric oxide release (data not shown); furthermore, addition of CAP18 or CAP18 peptides to supernatants from LPS-stimulated RAW 264.7 cells did not interfere with the ability to detect RNI in the Griess reaction (reference 21 and data not shown). CAP18 peptides did not inhibit generation of nitric oxide by murine gamma interferon alone (reference 21 and data not shown).

(iii) Human CAP18$_{104-135}$ inhibits LPS-induced tissue factor generation by macrophages. Previous studies demonstrated that unpurified rabbit CAP18 inhibited LPS-induced tissue factor expression by murine macrophages. Following identification of rabbit CAP18$_{106-142}$ as the active LPS-binding portion of rabbit CAP18, synthetic CAP18$_{106-142}$ and derivative peptides were demonstrated to inhibit LPS-induced generation of tissue factor by murine macrophages (17). The activities of human CAP18$_{104-140}$ and truncated CAP18$_{104-135}$ were compared with those of the rabbit peptides. Various concentrations of *S. minnesota* smooth LPS were incubated with each peptide at 37°C for 5 min prior to being mixed with thioglycolate-stimulated murine peritoneal macrophages. Production of tissue factor after 6 h was measured by a clotting assay. Figure 5 shows that the tissue factor induced in response to 0.1 and 1 μg of LPS per ml was inhibited by full-length rabbit but not full-length human CAP18 peptides. Previous work demonstrated that the non-LPS-binding peptides CAP18$_{105-114}$ and CAP18$_{117-142}$ do not inhibit LPS-induced tissue factor (17), and in the absence of LPS no tissue factor is synthesized by these cells. The truncated human CAP18 peptide (CAP18$_{104-135}$) demonstrated activity comparable to that of the rabbit peptide (Fig. 5).

In summary, the truncated human peptide CAP18$_{104-135}$ inhibits LPS induction of tissue factor at concentrations similar to those previously observed for the rabbit CAP18 peptides. Related cationic peptides without LPS binding activity do not inhibit LPS induction of tissue factor, and other control experiments demonstrated that CAP18 does not nonspecifically inhibit non-LPS stimuli of macrophages (data not shown).

Synthetic human CAP18$_{104-135}$ fragment inhibits LPS lethality in mice. Two murine models of endotoxemia were used to evaluate the capacities of human CAP18 peptides to neutralize two different types of LPS in vivo. Although rodents are relatively resistant to the lethal effects of LPS, pretreatment with galactosamine or actinomycin D augments their sensitivity. CAP18$_{104-135}$ attenuated the lethality of LPS to actinomycin D-sensitized (Table 3) and galactosamine-sensitized (Table 4) mice; however, CAP18$_{104-140}$ was not protective in these models, which is consistent with its relatively weaker anti-LPS activity in vitro (data not shown).

DISCUSSION

CAP18 was originally described as a cationic antimicrobial protein with a molecular mass of 18 kDa that agglutinated Re-LPS-coated erythrocytes and mediated bacterial cytolysis in vitro (48). Using as an assay the agglutination of Re-LPS-coated erythrocytes, we purified rabbit CAP18 and cloned the cDNA (22). To our surprise the sequence of the purified pro-

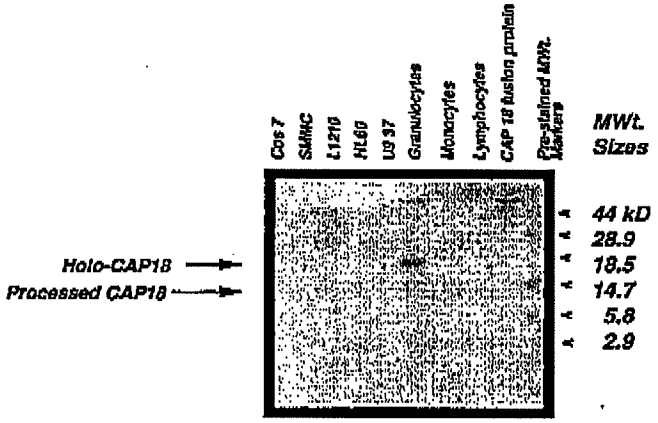

FIG. 3. Western blot demonstrating specific synthesis of CAP18 by human granulocytes. Soluble protein from approximately 20×10^6 cells electrophoresed on an SDS–12% PAGE gel was transferred onto nitrocellulose for immunoblotting with a CAP18-specific antiserum. Holo-CAP18 and the N-terminal fragment were specifically detected only in granulocytes. MWt., molecular mass.

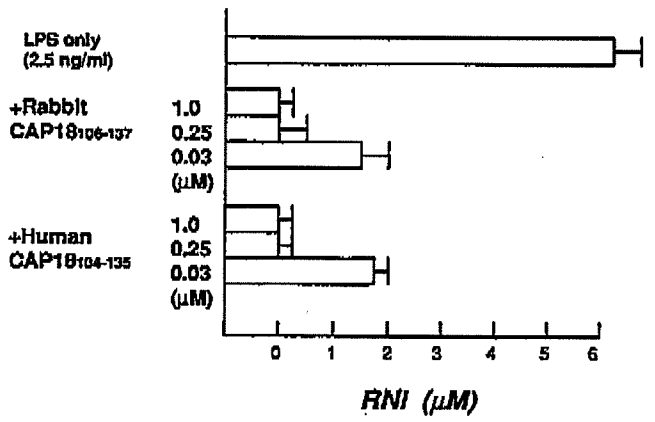

FIG. 4. Synthetic human CAP18$_{104-135}$ and rabbit CAP18$_{106-137}$ inhibit LPS-stimulated release of nitrogen radicals by murine RAW 264.7 macrophages. Inhibition of RNI released in response to 2.5 ng of LPS per ml by CAP18 peptides is shown.

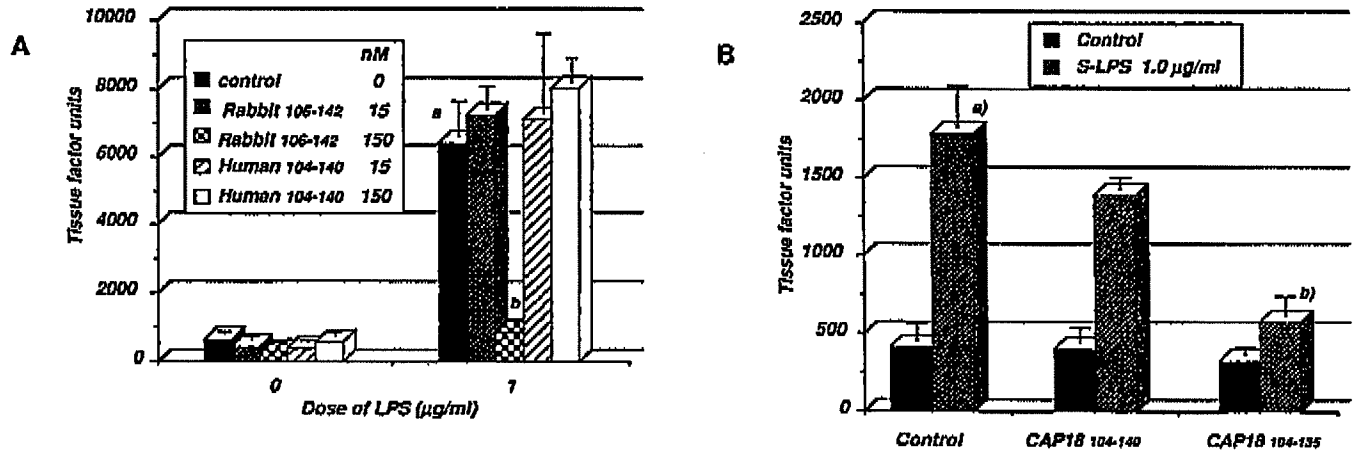

FIG. 5. Effects of human and rabbit CAP18 peptides on LPS-induced tissue factor generation. (A) Effects of CAP18 peptides. Full-length rabbit $CAP18_{106-142}$ peptide but not full-length human $CAP18_{104-140}$ peptide is active. S-LPS was incubated with each peptide at 37°C for 5 min, and then the mixture was added to a cell suspension and cultured for 6 h. a, $P < 0.05$ compared with the medium control; b, $P < 0.05$ compared with the LPS control. (B) Effects of human peptides. Truncated human CAP18 peptide $CAP18_{104-135}$ attenuated LPS-induced generation of tissue factor. S-LPS was incubated with each peptide (150 nM) at 37°C for 5 min, and then the mixture was added to a cell suspension and cultured for 6 h. a, $P < 0.05$ compared with the medium control; b, $P < 0.05$ compared with the LPS control.

tein corresponded to the C-terminal 37 amino acids of the protein (17, 21). Subsequent experiments confirmed that the C-terminal domain of CAP18 neutralized various activities of LPS and had potent antimicrobial activity (17, 20, 21). In the present work we cloned and sequenced the cDNA of human CAP18. By the translation of this cDNA, we identified and studied the corresponding C-terminal domain, $CAP18_{104-140}$. Western blot analysis using a high-titer anti-human CAP18 antiserum permitted identification of granulocytes as the primary cell type synthesizing human CAP18 in peripheral blood.

Since the publication of our original paper describing the cDNA for rabbit CAP18, several interesting sequences have been added to the databases, suggesting that CAP18 is a member of a novel family of proteins composed of two functional domains (9, 32, 42, 56). We hypothesized that the LPS-binding domain would be highly conserved and designed our initial cloning strategy accordingly. Unexpectedly, this domain has less than 40% amino acid identity among CAP18 family members, in contrast to the N-terminal domain, which has 60 to 70% homology. A more distant relative of the family (about 30% identity) was cloned by Levy et al. (25). This protein, also isolated from leukocytes, potentiates the antimicrobial activity of BPI. An interesting paper by Ritonja et al. (35) suggesting that porcine CAP18 was a cysteine protease inhibitor proved to be incorrect when this group subsequently reported identification of a stefin-like protease inhibitor active in the low picomolar range from pig leukocytes that probably contaminated their preparation of porcine CAP18 (24). Thus, at the present time the function of the more highly conserved N-terminal domain of CAP18 and related proteins is not known.

Several granulocyte antimicrobial proteins are derived from proproteins related to the conserved domain of CAP18. Two peptides termed bactenecins (abbreviated Bac5 and Bac7) are proline- and arginine-rich antibiotic peptides originally isolated from the large granules of bovine neutrophils (12). These peptides are stored as inactive probactenecins (57). Purified probactenecins do not display any antibiotic activity in vitro against organisms that are susceptible to the mature forms. Specific removal of the pro portion generates the C-terminal bactericidal proline- and arginine-rich domains. Specific cleavage at a valine adjacent to the Bac5 peptide is mediated by elastase, present in the azurophilic granules, and is triggered by neutrophil stimulation with bacteria (57). When the full-length cDNA of Bac5 was cloned, it revealed a protein composed of a 29-amino-acid signal and a 101-residue prosequence homologous to CAP18 (9, 56). The prosequence is acidic and may neutralize the highly cationic protein Bac5. The cDNA predicts the carboxyl-terminal tripeptide GRR which is missing from the carboxyl terminus of the mature peptide. The sequence GRR is a general proteolysis-amidation signal (27).

TABLE 3. Human $CAP18_{104-135}$ blocks LPS lethality in actinomycin D-treated mice[a]

Peptide	Amt of LPS (μg/mouse)	No. of mice dead/total	Survival (%)
None		0/7	100
None	1.0	10/11	9.1
$CAP18_{104-140}$ (1.0 μg/mouse)	1.0	7/7	0
$CAP18_{104-135}$ (1.0 μg/mouse)	1.0	2/7	71.4[b]

[a] Smooth LPS (*E. coli* O111:B4; 10 μg/ml) was incubated with an equal volume of each peptide (10 μg/ml) at 37°C for 30 min, and then the mixture (0.2 ml) was injected i.p. Actinomycin D (25 μg per mouse) was injected i.p.
[b] $P < 0.05$ compared with the LPS control.

TABLE 4. Human $CAP18_{104-135}$ blocks LPS lethality in galactosamine-treated C57BL/6 mice[a]

Amt of $CAP18_{104-135}$ (μg/mouse)	Amt of LPS (μg/mouse)	No. of mice dead/total	Survival (%)
		0/7	100
1		0/7	100
	0.1	13/16	18.8
0.1	0.1	8/12	33.3
1	0.1	4/12	66.7[b]

[a] Smooth LPS (*S. minnesota*; 1 μg/ml) was incubated with an equal volume of the peptide (10 or 1 μg/ml) at 37°C for 30 min, and then the mixture (0.2 ml) was injected i.p. Galactosamine was injected i.p. (15 mg/mouse).
[b] $P < 0.05$ compared with the LPS control.

A diverse group of porcine antimicrobial peptides, including PR-39 (42, 43), protegrin PG-2, and PMAP-23 and PMAP-36 (42, 43, 59), which like human CAP18 share a highly conserved N-terminal domain with cathelin (35), have been identified. The C-terminal peptide (20 amino acids) derived from the cDNA sequence isolated by Storici et al. (43) (PMAP-36) exhibited antibacterial activity particularly versus *Pseudomonas aeruginosa*. This peptide was shown to have an alpha-helical structure and to permeabilize the inner membrane of *E. coli*, characteristics shared with CAP18 peptides (43).

In addition to the CAP18 family other antimicrobial proteins isolated from granulocytes include the family of 30- to 35-amino-acid defensins (23), azurocidin (CAP37) (30), and BPI (10, 11, 40). A recent publication describes peptides derived from CAP37 which are active at concentrations approximately 2 to 3 log units higher than are the peptides derived from CAP18 described herein (30). The indolicidin peptides (39) and the defensins inhibit the growth of gram-positive and gram-negative bacteria only in hypotonic media, thus distinguishing them from $CAP18_{104-140}$ (19a). Because recent studies have demonstrated toxicity of neutrophil cationic peptides toward various eukaryotic targets such as *Giardia lamblia* (2), neuronal and glial cells (33), and lymphocytes (36), further studies of CAP18 toxicity are warranted. Future studies will investigate the relative roles of this diverse group of proteins in host defense and autoimmune injury.

LPS induces TNF and other mediators, such as nitric oxide, that produce deleterious effects on microvascular cells, contributing to capillary leakage, tissue injury, and multiple organ failure. Uncontrolled synthesis of nitric oxide may mediate TNF-induced hypotension (19). Rabbit $CAP18_{104-140}$ was originally identified and purified on the basis of its inhibition of LPS induction of nitric oxide synthetase in mouse macrophages (21). The present study demonstrates that human $CAP18_{104-135}$ binds to and inhibits LPS-induced generation of nitric oxide and tissue factor. Binding to LPS and inhibition of multiple LPS activities appear to be major functions of the C-terminal domain of CAP18, $CAP18_{104-140}$, and the truncated 32-amino-acid peptide, $CAP18_{104-135}$, is more active than the native peptide. The question of whether further proteolytic processing of the anti-LPS domain ($CAP18_{104-140}$) occurs naturally and the structural basis of the increased activity are currently under investigation.

The plasma of humans with sepsis seldom contains >1 ng of LPS per ml (29). In the direct inhibition of LPS-induced nitric oxide production, approximately 1 μg of $CAP18_{104-135}$ per ml was required to block the activity of 1 to 5 ng of purified LPS per ml. It is not clear why a large molar excess of the peptide is required to block activity in vitro (e.g., in the LPS-induced RNI assays), whereas a smaller molar excess is required in vivo. One possibility is that the peptide does not directly neutralize LPS in vivo but rather acts to clear LPS. Another possibility is that the peptide has other activities in vivo, such as modulation of blood coagulation (15a). Because limited toxicology studies indicated that $CAP18_{104-135}$ does not exhibit acute toxicity in mice when given at doses up to 20 mg/kg of body weight, it is likely that LPS released during sepsis in humans can be neutralized by achievable concentrations of CAP18 peptides. These findings suggest that this peptide may be able to attenuate LPS toxicity in humans.

New approaches for therapy of sepsis are urgently needed. Several endogenous (e.g., interleukin-1 receptor antagonist [1] and soluble TNF receptor [3]) and exogenous (e.g., anti-LPS antibodies and anti-TNF antibodies [7, 58]) agents have produced equivocal results in phase III trials. While these agents can prevent the action of individual members of the proinflammatory cytokine cascade, $CAP18_{104-140}$ presumably acts directly on gram-negative bacteria and LPS to block the primary stimulus for the entire cascade. Thus, initial studies of CAP18 (52) in porcine endotoxemia appear promising and further work to evaluate the therapeutic potential of CAP18 for treatment of the sepsis syndrome seems warranted.

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